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(54) Title: METHODS FOR MULTIPLE TARGET ANALYSES THROUGH NUCLEIC ACID HYBRIDIZATION

(57) Abstract

This invention provides for technical improvements for conducting nucleic acid hybridization assays. The improvements are designed to provide for a multiple target mode of conducting hybridization assays wherein a multiplicity of different nucleic acid probes for the site-specific capture of target nucleic acids are conjugated to specific locations upon a single dipstick or device. The use of a dipstick has substantial mechanical advantages over prior art formats for nucleic acid hybridizations. The dipsticks are composed of a handle, a nonporous support coated with a solid surface having at least one discrete region of nucleic acids covalently bound thereto. The improvements also provide means for covalently attaching nucleic acids to solid supports, improved hybridization buffers, improved support surfaces, methods for reducing nonspecific background and methods for quantifying assay results.

METHODS FOR MULTIPLE TARGET ANALYSIS THROUGH NUCLEIC ACID HYBRIDIZATION

BACKGROUND OF THE INVENTION

Field of the Invention

This invention provides for technical improvements for conducting nucleic acid hybridization assays. The improvements are designed to provide for a multiple target mode of conducting hybridization assays wherein a multiplicity of different nucleic acid probes for the site-specific capture of target nucleic acids are conjugated to specific locations upon a single dipstick or device. The use of a dipstick has substantial mechanical advantages over prior art formats for nucleic acid hybridizations. The dipsticks are composed of a handle, a nonporous support coated with a solid surface having at least one discrete region of nucleic acids covalently bound thereto. The improvements also provide means for covalently attaching nucleic acids to solid supports, improved hybridization buffers, improved support surfaces, methods for reducing nonspecific background and methods for quantifying assay results.

Information Disclosure

The nucleic acid hybridizations described herein require the immobilization of a capture nucleic acid to a solid support surface. Methods for the immobilization of nucleic acid to solid supports are known. Bischoff, R. et al., Introduction of 5'-Terminal Functional Groups into Synthetic Oligonucleotides for Selective Immobilization. Anal. Biochem. 164:336-344 (1987); Wolf, S.F. et al., Rapid Hybridization Kinetics of DNA Attached to Submicron Latex Particles, Nuc. Acids Res. 15:2911-2926 (1987); and J. N. Kremsky et al., Immobilization of DNA via Oligonucleotides Containing an Aldehyde or

effect the diffusion of the free nucleic acids and comprising at least one and preferably a multiplicity of discrete regions having nucleic acid probes covalently bound thereto. It is preferred that the thickness of the dipstick be between about .6 mm and about 10 mm, but more preferably between 1 and 2 mm. The dipstick is generally longer than it is wide. The portion of the dipstick immersed into test solutions is about 5 to about 10 times longer than it is wide. The discrete regions of nucleic acid are generally uniformly spaced apart and the regions may be either a rectangular or circular pattern on the solid surface.

The nonporous solid support can be of the same material as the handle and can be any size or shape. It is preferably elongated and adapted for dipping into small volumes of liquid. The solid support could be plastic, metal, or paper, and is preferably unchanged when exposed to hybridization media. By nonporous it is meant that nucleic acid in the sample solution does not become entrapped below the surface of the support. A solid support having extremely small pores would also fall within this functional definition as it would be effectively nonporous.

The solid surface is preferably selected from the following group: polystyrene/latex; polystyrene; immobilized latex beads; carboxyl modified latex microspheres; carboxyl modified glass; and carboxyl modified teflon. The surface may optionally be of the same material as the solid support.

The dipstick's solid surface preferably avoids a porosity which interferes with the diffusion rate of the free nucleic acids such that porosity is not a rate limiting factor in the nucleic acid hybridization assay. The avoidance of a rate limiting porosity can be achieved by selecting large or small pores. By "small," the porosity can be nonexistent (nonporous) or of such diameter that the surface is effectively nonporous. The

proteins include thiolated bovine serum albumin; casein; and liquid gelatin.

This invention also provides for a dipstick as described above wherein the nucleic acid probes are complementary to regions of RNA found within ribosomes including both the 16S and 23S RNA. The nucleic acid probes may be complementary to either hypervariable or conserved regions of the ribosomal RNA.

This invention also provides methods for assaying the presence of target nucleic acids, the method comprising contacting a hybridization medium containing target nucleic acids with a dipstick comprising a solid surface having a multiplicity of discrete regions with different nucleic acid probes covalently bound thereto. By "different" it is meant that the nucleic acid probes do not have identical nucleic acid sequences and will preferentially bind to different target nucleic acid. This method is preferably conducted using the solid surfaces described above for the dipstick and is most preferably conducted using carboxyl modified latex microspheres. The surface for conducting this method preferably does not inhibit the diffusion of nucleic acid. Most preferably, the surface has a porosity of the size ranges described above for the dipstick.

The method disclosed herein is preferably conducted using nucleic acid probes that are covalently bound to the solid surface through spacer arms. The spacer arms are as described above for the dipstick.

In a preferred mode of operation, the disclosed method has proteins conjugated to the solid surface through sulfhydryl bonding. The preferred proteins are as described for the dipstick. This method is preferably useful when the nucleic acid probes are complementary to sequences of nucleic acid indicative of pathogenic state in mammals. More preferred are nucleic acid probes which are complementary to sequences of RNA found within ribosomes. The preferred ribosomal RNA and the preferred

tetramethylammonium chloride or tetraethylammonium chloride. The preferred concentration of tetraalkylammonium salt is between about 2 and 3.5 moles per liter.

The method and the dipstick can be surface modified by covalently binding a surface-modifying moiety through a covalent bond selected from the group consisting of disulfide linkage and thiol ether. The surface-modifying moiety can be hydrophilic, hydrophobic, ionic or metallic. By hydrophobic it is meant that the dielectric constant for the relevant moiety is below 30, preferably between 1 and 30, and by hydrophilic it is meant that the dielectric constant is above 30, preferably between 30 and 80. Metallic surface-modifying moieties include copper, gold, iron, chrome, silver and aluminum. The preferred surface-modifying moiety is of the formula:

$$-C{:}O-N(H)-R_{12}-S-S-R_{13}$$

wherein R_{12} and R_{13} are different and are comprised of organic residues. These organic residues are non-critical features of this invention. There is virtually no limit to the substitutions which could be made so long as the ability to retain the thiol ether or thiol linkage is preserved. Practical limits preclude molecular weights in excess of 1000 daltons. The specific substituents for R_{12} and R_{13} will vary in accordance with the needs and convenience of the situation. With the understanding that R_{12} is a divalent radical, such as methylene, and R_{13} is a monovalent radical, preferred substituents include for R_{12}: methylene [$-(CH_2)_n-$], (C1-C10); alkylaryl (C7-C14); aryl (C6-C10); and for R_{13}: alkyl (C1-C10); alkylcarboxyl (C1-C10); aryl (C6-C10); aminoalkyl (C2-C10); N-alkylaminoalkyl (C3-C15), N,N-dialkylaminoalkyl (C4-C15); substituted aryl (C6-C9) wherein the substituents are the same or different, the number of substituents are 1-3 and are alkyl (C1-C3), amino, N-alkylamino, N,N-dialkylaminoalkyl, thio, carboxyl, nitro, sulfo, halo, acyl of $R_{14}-C=O-$ or

targets and bacterial targets (particularly the ribosomal RNA). When conducting the assay in the multiple target mode, it is preferred that the ammonium salts previously described are used when needed to offset different GC contents between the reactants.

When using a dipstick or other solid support and when conducting the disclosed methods, it is preferred that the solid support include surface-modifying moieties bound to surface through a covalent bond selected from the group consisting of disulfide linkage and thiol ether. These surface-modifying moieties are as previously described.

There is also described herein a method for decreasing the sensitivity of a nucleic acid hybridization assay by a known amount, the method comprising adding a predetermined ratio of unlabeled polynucleotide signal probes to labeled polynucleotide signal probes, permitting the signal probes to bind to their complementary sequences, and detecting hybridization of the labeled signal probe. This permits one to adjust the sensitivity of an assay to permit sensitivity cutoffs to be set. Sandwich assays are a preferred format for reduction of sensitivity. Bacterial nucleic acid is the preferred target. Kits having containers of signal probes with varying ratios of label to unlabeled probes are also described.

Throughout this document the terms nucleic acid, polynucleotide and oligonucleotide are interchangeable except where a specific size range is indicated by language context.

DETAILED DESCRIPTION

This invention discloses a nucleic acid hybridization assay format which uses a dipstick. The dipstick format has mechanical advantages over other formats presently being used. It permits ready agitation without additional mechanical devices. The handle

include fecal samples, blood, sputum, saliva, urine, semen, plaque samples, tissue samples, and the like. The methods of the present invention will not differ regardless of whether exogenous or endogenous nucleic acid is being detected and hence the simultaneous detection of viral, bacterial or host nucleic acid is made possible.

Pathogenic viruses and microorganisms which may be detected in fecal or gastric samples, or as contaminants of foods, beverages or water, or the like, and include viruses, such as adenovirus, rotavirus and the like; bacteria, such as Salmonella spp. (S. typhimurium, S. typhi, S. paratyphi A, etc.), Shigella spp. (S. dysenteriae, S. flexneri, S. boydii, S sonnei, etc.), Campylobacter spp. (C. jejuni, C. coli, C. pylori), Clostridium difficile, Escherichia coli, Yersinia enterolitica, and the like; fungi, such as Candida albicans, and the like; and protozoans, such as Giardia lamblia, Entamoeba histolytica, Microsporidium spp., and the like.

Pathogenic viruses and microorganisms infecting the lung, bronchial and upper respiratory areas which may be detected in saliva, sputum or respiratory lavage samples include viruses, such as adenovirus, respiratory syncytial virus, human papillovavirus, human immunodeficiency virus, human T-cell lymphotrophic viruses, cytomegalovirus, hepatitis A and B virus, epstein-barr virus, and the like; bacteria, such as Streptococcus pyogenes (Group A beta-hemolytic streptococci), Haemophilus influenzae, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa, Neisseria meningitidis, Mycobacterium tuberculosis, Spirochaetales, such as Treponema spp., and the like; fungi, such as Candida albicans, Histoplasma capsulatum and the like; and protozoans, such as Pneumocystis carinii, and the like.

are provided. To avoid nonspecific binding of target nucleic acid, a solid surface capable of being chemically cleaned by disulfide reduction is provided. To permit the use of a universal hybridization media, the addition of alkylammonium salts is provided. The use of solid supports having a porosity which does not inhibit the diffusion of the target nucleotides is also disclosed. Finally, a method for controlling the sensitivity of the assay is provided.

I. GENERAL METHODS FOR NUCLEIC ACID HYBRIDIZATION

This invention relates to nucleic acid hybridization assays which allow for the detection of multiple target nucleic acids (i.e., pathogens) on a single dipstick. Accepted means for conducting such assays in a single target mode are known and general overviews of the technology can be had from a review of Nucleic Acid Hybridization: A practical Approach, Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1985; Hybridization of Nucleic acids Immobilized on Solid Supports, Meinkoth, J. and Wahl, G, Analytical Biochemistry, Vol. 238, 267-284, 1984 and U.S. Patent No. 4,358,535 which are incorporated herein by reference.

A. Sample Collection and Preparation

Target polynucleotides can be obtained from a variety of biological sources, depending upon the particular target one desires to detect. Sources include all manner of biological materials which may harbor polynucleotide targets. Polynucleotide targets can be: endogenous nonpathogenic nucleic acid sequences; mutations of the normal wild-type population, regardless of whether they are phenotypically expressed; or nucleic acid sequences arising from the presence of pathogens, such as viruses, bacteria, mycoplasmas, protozoa, rickettsia or fungi.

sequence or portions thereof of an organism's genome, from messenger RNA, or from cDNA obtained by reverse transcription of messenger RNA. After isolation of genomic DNA or cDNA fragments, the fragments are typically inserted into a replication vector, such as lambda phage, pBR322, M13, or vectors containing the SP6 or T7 promoter and cloned as a library in a bacterial host. Following appropriate screening procedures, a recombinant vector with the desired probe insert is isolated and labelled as described below. The vector is then grown in a suitable host. The probe and its vector are purified from the host cells by cell lysis and nucleic acid extraction. Following isolation, the probe can be purified away from the vector by digestion with selected restriction enzymes and sequenced. Further isolation of the probe can be achieved by using gel electrophoresis or high pressure liquid chromatography.

Once the appropriate sequences are determined, DNA probes are preferably chemically synthesized using commercially available methods and equipment. For example, the solid phase phosphoramidite method can be used to produce short probes of between 15 and 50 bases and have a molecular weight of less than 16,000 daltons. These are referred to herein as "short probes." (Caruthers, et al., Cold Spring Harbour Symp. Quant. Biol., 47:411-418, 1982, and Adams, et al., J. Am. Chem. Soc., 105:661, 1983).

When synthesizing a probe for a specific target, the choice of nucleotide sequence will determine the specificity of the test. For example, by comparing DNA sequences from several virus isolates, one can select a sequence for virus detection that is either type specific or genus specific. Comparisons of DNA regions and sequences can be achieved using commercially available computer programs.

Probes may be labelled by any one of several methods typically used to detect the presence of hybrid

ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labelled, naturally occurring anti-ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

Probes can also be conjugated directly to signal generating compounds, e.g., by conjugation with an enzyme or fluorophore ("labeled signal probes"). Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol.

C. Hybridization Conditions

Various hybridization solutions may be employed, comprising from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs about 30-60% v/v formamide, about 0.5 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris HCl, PIPES or HEPES, about 0.05 to 0.5% detergent, such as sodium dodecylsulfate, and between 1-10 mM EDTA, 0.01 to 5% ficoll (about 300-500 kilodaltons), 0.1 to 5% polyvinylpyrrolidone (about 250-500 kdal), and 0.01 to 10% bovine serum albumin. Also included in the typical hybridization solution will be unlabelled carrier nucleic acids from about 0.1 to 5 mg/ml, e.g., partially fragmented calf thymus or salmon sperm, DNA, and/or partially fragmented yeast RNA and optionally from about 0.5 to 2% wt./vol. glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as anionic polyacrylate or polymethylacrylate, polystyrene sulfonic acid and charged saccharidic polymers, such as dextran sulfate.

After hybridization at a temperature and time period appropriate for the particular hybridization solution used, the glass, plastic, or filter support to which the probe-target hybrid is attached is introduced into a wash solution typically containing similar reagents (e.g., sodium chloride, buffers, organic solvents and detergent), as provided in the hybridization solution. These reagents may be at similar concentrations as the hybridization medium, but often they are at lower concentrations when more stringent washing conditions are desired. The time period for which the support is maintained in the wash solutions may vary from minutes to several hours or more.

Either the hybridization or the wash medium can be stringent. After appropriate stringent washing, the correct hybridization complex may now be detected in accordance with the nature of the label.

The probe may be conjugated directly with the label. For example, where the label is radioactive, the dipstick with associated hybridization complex substrate is exposed to X-ray film. Where the label is fluorescent, the sample is detected by first irradiating it with light of a particular wavelength. The sample absorbs this light and then emits light of a different wavelength which is picked up by a detector (Physical Biochemistry, Freifelder, D., W.H. Freeman & Co., 1982, pp. 537-542). Where the label is an enzyme, the sample is detected by incubation with an appropriate substrate for the enzyme. The signal generated may be a colored precipitate, a colored or fluorescent soluble material, or photons generated by bioluminescence or chemi-luminescence. The preferred label for dipstick assays generates a colored precipitate to indicate a positive reading. For example, alkaline phosphatase will dephosphorylate indoxyl phosphate which then will participate in a reduction reaction to convert

a one-step assay for the detection of a single pathogen within a sample. No description was given of the potential for the detection of multiple pathogens within a single test sample using DNA/RNA probes in a sandwich type of format.

The sandwich hybridization format using DNA probes provides a rapid diagnostic method which allows the detection of virtually any pathogen in crude cell lysates without prior isolation of the nucleic acid. In this format, two non-overlapping sequences complementary to the target nucleic acid are identified and at least two DNA/RNA probes are cloned or synthesized.

The first DNA probe subsequently is immobilized to a solid support and, when conducting a clinical assay, is used to capture a target nucleic acid from a complex cell lysate via hybridization to form a specific duplex. At the same time or at a subsequent time, the second DNA probe also hybridized with the target nucleic acid to form a specific duplex. This second DNA probe is labeled and can be detected directly through a probe-linked label such as an enzyme, or indirectly through a biotin:labeled-streptavidin system. The amount of the target nucleic acid (i.e., the pathogen) present in the clinical sample is indicated by the amount of labeled, second DNA probe which is contained within the hybridization complex.

B. Assay Discrimination is Achieved by Site-Specific Sequestering.

The sandwich format can be used to develop assays which detect multiple targets simultaneously. A principle feature of this assay is that discrimination of multiple nucleic acid targets (DNA and/or RNA) is achieved by site specific sequestering on a solid surface of the target nucleic acid itself.

An example of a multiple target assay for each of the 16S ribosomal RNA (rRNA) of three microbes is shown

preclude the use of enzymes as labels unless appropriate inhibitors are added after lysis. However, the second DNA probe can be labelled with biotin. After hybridization and removal of proteases and/or chaotropic agents by a washing step, the hybridized biotin-labeled DNA probe can be detected with avidin conjugated with a signal generating system as described below for the two-step assay.

ii. Two-Step Hybridization Assay

In the two-step hybridization assay, the hybridizations of the unlabeled, immobilized probe and the labeled probe with the target nucleic acid are conducted separately. For example, the samples can be mixed with disruption agents to lyse cells and release nucleic acid, and the unlabeled, surface-immobilized probe can be immersed such that hybridization proceeds in the presence of the disruption agents. After the sequestering of the target nucleic acid on the surface, the surface is washed and the second hybridization is allowed to proceed between the sequestered target nucleic acid and the labeled probe in solution.

The two-step assay is required when the assay uses a probe labeled with an enzyme which would be deleteriously affected by proteases or chaotropic agents in the lysing buffer. In this case, a second hybridization is conducted in a medium which will be compatible with the maintenance of activity of the enzyme linked to the probe.

The two-step assay also can be run in an alternative mode -- after cell lysis, the target nucleic acid can be hybridized first with the signal-generating probe, and second with the immobilized probe.

A number of important characteristics of the multiple target dipstick assay used in either the one-step or two-step modes should be pointed out:

TABLE 1

Assay Type	Capture Probe	Signal Probe
a) One/Two step	Specific	Specific*
b) One/Two step	Specific	Non-Specific*
c) One/Two step	Partially Specific	Specific*
d) One/Two step	Non-Specific	Specific

*Preferred

When conducting the multiple target assay, it may be necessary to use a signal-generating probe which is highly specific for its target 16S rRNA. This specificity will be important if the immobilized probe exhibits some cross-hybridization with heterologous 16S rRNAs or other RNAs or DNAs.

In contrast, if the immobilized probe is highly specific for its target 16S rRNA, it will not be necessary to have a highly specific labeled probe. In this case, the labeled probe can be designed to hybridize with a conserved region of 16S rRNAs. such a probe will hybridize effectively with all microbial 16S rRNAs and, in effect, will detect the presence of any 16S rRNA site-specifically sequestered on a surface during the multiple target assay.

Finally, it will be possible to use multiple, labeled probes simultaneously for the detection of multiple different conserved regions of 16S rRNA. This feature has two implications. First, it will allow the development of more highly sensitive multiple target assays since many different labeled probes can be used, each complementary to a different conserved region of 16S rRNA. Second, it will be possible to develop a generic

often times involve nucleic acids having significant variations in their respective G:C and A:U or A:T ratios. The influence upon hybridization due to differing compositions is most readily viewed between nucleic acids having the same length. The G:C hydrogen bonding is more stable under typical salt conditions than the A:U or A:T bonding. Therefore nucleic acids having significantly different compositions will have significantly different ability to stably hybridize under similar hybridization conditions.

Tertiary and quaternary alkyl ammonium salts are able to stabilize the A:T base pair to the point where it is equivalent to the stability of the G:C base pair. The cations of these salts include akyls of 1-4 carbons, inclusive, with both branched and straight chain. The individual alkyl substituents may be the same or different. The anions are non-critical and include acetate, iodide, perchlorate, thiocyanate, chloride and bromide and the like. The most preferred anions are bromide or chloride. The most preferred salt is tetraethylammonium chloride which works best at 30-40°C in concentrations of between 2 and 3 moles per liter most preferably about 2.4 moles per liter. The preferred nucleic acid chain length is about 15-50 nucleotides most preferably about 24 nucleotides. The effects of various alkylammonium salts upon nucleic acid hybridization are known and generally reviewed in Biopolymers 16:1183-1199 (1977).

ii. Cutoff Limits

Another feature of the multiple target assay is that it will be possible to set cutoff limits for the detection of individual microbes in the assay. Cutoff limits will be important for the detection of contaminants or diseases which are caused by, or diagnosed by, the overabundance of a etiologic agent such as microbes or fungi. The most straight forward method

To illustrate clinical application, it is known that several microbes are associated with bacterial vaginosis which is a vaginal infection curable by treatment with the proper antibiotics. In the disease condition these organisms are typiclally present at different abnormally high levels. Using the procedures described above, a diagnostic assay based upon visualization of color can be constructed using either the single or multiple target modes, preferably the multiple target mode. In such assays, it will be possible to set cutoff limits such that a positive test (eg., a detectable or specific shade of color on the dipstick) for a disease state will be indicated only when the concentration of a microbe exceeds a certain value.

To further illustrate the usefulness of the cutoff limits, assume that an infection becomes clinically important when 10^6 molecules/ml of that microbe's 16S rRNA are present in a patient sample. Also, assume that the use of a labeled probe (without dilution) can just detect 10^4/ml of that microbe's 16S rRNA using the deposition of colored precipitate as an indicator. To achieve a simple cutoff limit, wherein the test only produces a positive result when there are 10^6/ml molecules of that microbial 16S rRNA, it will theoretically only be necessary to mix one equivalent of labeled probe per 99 equivalents of unlabeled probe. This will decrease the sensitivity of the assay 100 fold, and will move the cutoff limit for molecules of that microbe's 16S rRNA from 10^4/ml to 10^6/ml. These ratios assume that the labelled and unlabelled probe hybridize at the same rates and form stable duplexes with the target. In practice the rates may differ and the setting of appropriate cut off limits will require empirical determination of the ratios of labeled to unlabelled probe.

This type of label dilution can be used to desensitize virtually any assay and can be individually

sulfhydryl groups for subsequent reaction with the thiol-reactive oligonucleotide described below in section II part B. The preferred method is to modify the surface with disulfide derivatives containing nucleophilic amines which participate in conjugation, and then to reduce the disulfide groups to monothiols by treatment with, for example, dithiothreitol, in order for the thiol reactive portion of the activated oligonucleotide to covalently bind to the surface.

Virtually any solid surface can be used in this invention, including metals and plastics. Three types of solid surfaces are available for modification, namely:

a) Membranes, Nytran® (Schleicher & Schuell, Inc., Keene, NH 03431) polystyrene beads, teflon, polystyrene/latex beads, latex beads or any solid support possessing an activated carboxylate, sulfonate, phosphate or similar activatable group are suitable for use as solid surface substratum.

b) Membranes, polystyrene beads, teflon, polystyrene/latex beads, latex beads or any solid support possessing an activated carboxylate, sufonate or phosphate are suitable for use as solid surface substratum.

c) Porous membranes possessing pre-activated surfaces and may be obtained commercially (eg. Pall Immunodyne Immunoaffinity Membrane, Pall, Pall BioSupport Division, East Hills, NY, or Immobilon Affinity membranes from Millipore, Bedford, Mass.)

The following chemical moieties can be attached to the solid support surface: any aminoalkyl or aryl disulfides (for coupling of the oligonucleotides), any mercaptans (for surface modification), any amino thiol alcohols (for coupling of oligonucleotides), amino aryl disulfides (for coupling of the oligonucleotides), any NH2-R12-S-S-R13-SH where R12 and R13 are the same or different. R_{12} and R_{13} are noncritical moieties that are not to be viewed as a limitation of this invention. They

plastic, glass or ceramic support with the use of appropriate adhesives.

In the case of spheres or beads consisting of Nytran®, polystyrene, polystyrene/latex, latex, or any other polymer possessing an activatable carboxy group, the procedure of J.V. Staros et al., Analytical Biochemistry 156:220-222 (1986) is employed. The beads are placed in a 100,000 to 1,000,000 molar excess of 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC), or other carboxyl activating reagent, in aqueous buffer at pH 5.0 to 5.5 for 2 minutes at 20° to 30° C. The excess and unreacted EDC is then removed by centrifugation or filtration of the beads. The activated beads are exposed to 0.01 to 1 molar solution of an aminoalkyldisulfide or an aminoalkylmercaptan preferably cystamine or cysteamine (2-aminoethanethiol) in a non-amine containing buffer such as sodium borate, sodium phosphate or sodium carbonate at about pH 7 to 9 and allowed to react for 1 to 24 hours.

After appropriate washing steps, followed by centrifugation or filtration, the disulfide of the conjugated cystamine is reduced to a sulfhydryl using a reducing agent such as 0.01 to 0.1 M dithiothreitol or dithioerythritol If cysteamine was employed in the last step, the beads do not require a reduction step.

Support surfaces described above without activatable carboxylate groups can also be activated. These supports can be any shape or size. The surfaces are first coated with a solution of latex polymer which contains carboxyl groups (Carboxylate-Modified Tube Coating (CML), Seradyne, Inc. Indianapolis, IN). Following drying, the carboxyl groups on such coated surfaces can be activated, and then conjugated with modified oligonucleotides as explained below in section IV part C. The use of the CML coating is a preferred mode of producing a multiple target dipstick.

iii. Microtiter Plates and Wells

In the case of plastic 96, 48, 24 or 8 well microtiter plates or individual microtiter wells or well strips, CML Coating is applied and processed as described above and to each well a different or the same type oligonucleotide sequence may be covalently immobilized. This permits the formation of a device in which 96, 48, 24, or 8 different specific oligonucleotides may be immobilized and used in hybridization assays.

The solid surfaces now contain a sulfhydryl group ready to be activated with a thiol reactive oligonucleotide as described below in Section IV, Part C.

B. Functionalization of the Capture Oligonucleotide

The preferred capture nucleic acids for use in this invention are synthetic oligonucleotides of between 20 and 100 bases. During synthesis a linker arm containing a blocked amine group can be coupled using conventional chemistry to the 5'-hydroxyl group of an oligonucleotide. The activated oligonucleotides used as starting materials for this invention can be derived through several methods. The reagents for the attachment of primary linker arms terminating in a primary amine are commercially available. A primary amine is the preferred group for attachment to the heterobifunctional reagent, and its attachment via a hexyl arm is preferred. Starting materials suitable for use in this invention are described in PCT U.S. 86/01290; Nucl. Acids Res., 15:3131 (1987); Nucl. Acids Res., 15:2891 (1987); and Nucl. Acids Res., 14:7985 (1986).

for example, butane diamine to form a Schiff base, and 3) treating with sodium borohydride or cyanoborohydride to form a stable reduced Schiff base derivative in which one of the amines is left free for subsequent conjugation.

DNA bases can also be modified to become thiol reactive before or after preparation in a DNA synthesizer. Using the post-synthesis approach, natural nucleic acids (DNA and RNA) and molecularly cloned nucleic acids, as well as synthetic nucleic acids, can be modified. In the case of cytosine, a more nucleophilic amine is linked to the 4-position of cytosine by a variety of chemistries. The amine can be added by treatment with hydrazine to generate N-4-aminocytosine (Sverdlov, E.D., et al., FEBS Letters, 62, p. 212. Feb. 1976). This reaction is catalyzed by bisulfite. Alternatively, an amine can be added to the 4-position by bisulfite catalyzed transamination reactions where a diaminoalkane is added to the 4-position (Shapiro and Weisgras, Biochemical and Biophysical Research Communications, 40:839, 1970). Bi-functional semicarbazide can also be used to add nucleophilic amines to the 4-position of cytosine (Hayatsu and Ukita, Biochemical and Biochemical Research Communications, 14:198, 1964). The base with the free amino group in the oligo is then made thiol reactive through the reaction of the nucleophilic amine and a NHS ester of a carboxylic acid derivative substituted with a alpha- or beta-unsaturated carbonyl, or alpha-halocarbonyl groups or the like.

Other nucleic acid bases with nucleophilic amines on other positions can be used for reactions with NHS esters of a carboxylic acid derivative substituted with a alpha- or beta-unsaturated carbonyl, or alpha-halo-acyls. For instance, 5-[N-(7-aminohexyl)-1-acrylamido]-2'-deoxyuridine 5'-triphosphate (Calbiochem, La Jolla, CA) can be added to the 5' end of oligonucleotides in reactions with terminal

this invention can be purchased from Pierce Chemical Co., Rockford, IL. Examples include:

N-Succinimidyl 4-(iodoacetamido)benzoate (SIAB).

Sulfosuccinimidyl (4-iodoacetamido)benzoate (Sulfo-SIAB)

C. Conjugation of the Activated Oligonucleotide to the Thiolated Solid Support Surface

The thiolated surface of section IV, part A, is reacted with the activated oligonucleotide, using a 5 to 100-fold excess of activated oligonucleotide over the maximum oligonucleotide binding capacity of the solid support. This method gives 5 to 100 micrograms of oligonucleotide (for a 24-mer) per cm^2 for a Pall Immunodyne membrane or 0.05 to 1 microgram per cm^2 for undiluted Seragen CML Tube Coating.

The reaction occurs in aqueous buffer between ph 5.5 and 9.5 (with the pH range of 7-8 being preferred) and is allowed to proceed from 1 to 16 hours at about 19° to 24°C. Volumes of the reaction are held to a minimum. The conjugate is separated from free oligonucleotide by repeated washings in buffers containing ionic detergents such as sodium dodecyl sulfate, sodium laurylsulfate or hexadecyltrimethylammonium bromide (CTAB) or nonionic detergents such as Tween 20, with a final wash in water. The solid supports can be stored dry. The supports are stable indefinitely when stored dry or stored in the appropriate buffers at 4°C.

Oligonucleotides modified with thiol-reactive groups can be conjugated to the derivatized solid support by a variety of means. These include slow filtration in the case of membranes, immersion in a solution of functionalized oligo when plastics frits are used or overlaying the activated oligo solution on the activated surface of a flat plastic, glass or ceramic sheet.

Once the oligonucleotide is covalently bound to the solid support surface it is useful as a probe in

D. Postmodification of the Surface of the Solid Support

After conjugation of the surface of the solid support with a capture DNA probe, there is a large excess of free sulfhydryl groups. The ratio of these remaining sulfhydryl groups to covalently coupled capture oligonucleotide can range from 10:1 to 1,000,000:1. The presence of these excess thiol groups permits a second modification of the surface of the solid support by the selective chemical coupling of R-SH groups, where R is any chemical functionality. This chemical coupling can be either irreversible (e.g., by reaction with a maliemide derivative) or reversible (e.g., by formation of a disulfide bond).

The ability to conduct a second modification of the surface of the solid support is important for achieving a variety of desired surface properties on the solid support. The following are illustrative of some possible examples:

1. The thiolated surface provides the chemical means for irreversible modification in the second conjugation step by treatment modification of the residual thiols with the appropriate reagent to provide covalent bonds. Such conjugations can modify the charge, hydrophobicity, hydrophilicity, color, reflectance, transmittance, porosity, frictional coefficient, porosity, conductivity and heat capacity of the surface. The thiolated surface also provides the means whereby proteins, nucleic acids, antibodies, antigens or other macromolecules of interest may be irreversibly coupled to the solid support. Such modified surfaces will control, in part, the rate of hybridization of target nucleic acid to the capture probe on the solid surface support and will be important in reducing the level of nonspecific background arising during the detection of the hybridized labelled probe.

2. In contrast to the irreversible modification described directly above in Section 1, the thiolated

cleaved and the protein is released into the solution with its non-specifically associated labeled DNA probe.

It should be noted that this type of "chemical cleansing" (disulfide reduction to remove specific conjugated groups), in addition to removing background signal, can also be used to alter the charge, hydrophilicity, hydrophobicity, color, reflectance, transmittance, friction coefficient, porosity, conductivity and heat capacity of the surface of the solid support.

The following serves to illustrate more specifically the reversible "chemical cleansing" described directly above. After the activated oligonucleotide is irreversibly coupled to the support surface, the surface is placed in a neutral or basic buffer (if aqueous conditions are required), or organic solvent containing 0.0001-0.1 M thiolated bovine serum albumin (BSA) with 0.001-0.1 M iodosobenzoate, and mixed for 10 to 60 minutes at 15 to 50°C. The iodosobenzoate promotes an oxidation reaction which produces a disulfide bond between the dipstick and the BSA. Excess reagents are removed by alcoholic washes followed by aqueous washes containing ionic detergents. Surfaces modified in this way contain irreversibly conjugated capture probe and reversibly conjugated BSA-protein.

After formation of the hybridization complex between immobilized probe, target nucleic acid and labeled probe, all disulfides on the surface may be cleaved by treatment with dithiothreitol, beta-mercaptoethanol or the like. This highly specific cleavage removes much of the labeled DNA probes which is non-specifically bound to the dipstick surface. Such treatment allows the development of DNA probe assays which have high signal to noise ratios, and hence are extremely sensitive.

This cleansing is particularly advantageous where the target nucleic acid to be detected is present within

ceramics in any conformation or shape amenable to the desired dipstick format. Examples include porous polypropylene frits or porous glass, and nonporous material such as flat polystyrene sheets, microtiter plates and wells, glass tubes and the like.

The rate of hybridization of nucleic acids in solution to a complementary nucleic acid immobilized on a solid support is normally a three step process depending on the nature of the solid support. If the solid support is a filter membrane such as those commercially available from Pall or Millipore, the rate limiting steps leading to hybridization consist of the following:

1) The rate of external diffusion of the target nucleic acid to the dipstick device surface;

2) The rate of internal diffusion of the target nucleic acid within the solid support.

3) The rate of nucleation between the target nucleic acid and the immobilized capture probe.

In contrast to solution-phase nucleic acid hybridizations, which are only marginally rate limited by diffusion, the rate of hybridization of nucleic acids in solution to a solid support (mixed phase) is substantially limited by external and internal diffusional control. Therefore, to maximize the efficiency and rate of hybridization, several key parameters of the sandwich assay system have been defined. First, the concentrations of the nucleic acids should be kept as high as possible. Second, the volumes should be minimized to the fullest extent. Third, the distances should be minimized between the surface and the target nucleic acid in solution. Fourth, we have found that polymeric forms of capture nucleotides will provide good kinetics for hybridizing to target probes. The capturing polymers are then sequestered on the dipstick. The preferred polymer is polyethyleneamine having capturing oligonucleotides covalently bound thereto.

Minifold 11 apparatus (S&S Inc., Keene, N.H.) and 5 X 10^{-10} moles to 2.5 X 10^{-7} moles of cystamine (Aldrich Chemical Company, Milwaukee, Wisconsin) in 10 µl microliters was applied to the membrane in successive slots. The membrane was removed and washed 4 to 5 times with 15 to 25 ml of PBS (0.01 M sodium phosphate pH 7.4, 0.13 M NaCl) and then reduced with 0.1 M dithiothreitol (DTT) in PBS (15 to 25 ml) for 30 minutes with constant shaking. The membrane was washed 3 to 5 times with PBS and the appropriate slots cut out for analysis. The filter was then placed in 0.5 ml of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB, Pierce Chemical Company, Rockford, Illinois) for 15 minutes with constant shaking. 200 µl of the solution was then measured for absorbance at 405 nm to determine the degree of derivatization. The results were compared to a DTNB standard curve generated using DTT. The results indicated that between 7.5 X 10^{-12} and 2.25 X 10^{-11} moles of cystamine were covalently bound and reduced to 2-aminoethanethiol per 7 mm^2, respectively.

B. Binding of Cystamine to Polystyrene Beads or Polystyrene/Latex Beads

To demonstrate the binding of cystamine to polystyrene beads or polystyrene/latex beads (Interfacial Dynamics Corporation, Portland, Oregon) 200 microliters of beads (4.1% solids) were pelleted at 10,000g and then resuspended in 500 µl of 50mg/ml EDC in PBS at pH 5.5 and incubated 5 minutes at 22°C. The beads were then pelleted at 10,000 X g and resuspended in 50mg/ml cystamine in 0.05 M Na borate pH 8.3 and incubated 60 minutes at 40°C. The beads were then washed extensively with PBS containing 0.1% Tween and then reduced with 0.1 M DTT for 30 minutes at 22°C and again extensively washed with PBS/Tween. The DTNB assay (described above) indicated 5 X 10^{-11} moles of cystamine were bound (and reduced) to the beads. Cystamine binding to the Carbox-Modified Tube Coating (CML, Seragen Diagnostics, Inc. Indianapolis,

for 1 hour in the dark at ambient temperature. The excess reagent is removed by desalting (size exclusion) on a G-25 Sephadex column in PBS or PBS containing 3M NaCl.

Example 3: Conjugation of the Activated Oligonucleotide to the Solid Support.

The activated oligonucleotide can be bound to the solid support by two methods: (a) immersion if polystyrene/latex beads, CML coated surfaces or porous membranes are employed or (b) filtration if porous membrane is used. In this example, oligonucleotide binding was monitored by ^{32}P radioactivity as the oligonucleotide was labelled at the 3' end with ^{32}P-cordycepin phosphate. 0.12 to 1.2 μg of Bg5B6n was applied in 1 to 10 microliter volumes by filtration to 2-aminoethanethiol derivatized Pall Immunoaffinity membrane in a slot blot apparatus. The filters were washed extensively with an aqueous solution containing 0.5% sodium dodecyl sulfate (SDS) and then radioactivity was monitored by scintillation counting. The results indicate that up to 0.1-1.0 μg of oligonucleotide can be bound per 0.28 cm^2 of Pall membrane.

When the same SIAB-oligonucleotide was incubated by immersion with CML coated frits derivatized with 2-aminoethanethiol, up to 0.02 μg of DNA was bound to the frit. Similar results were obtained with derivatized polystyrene/latex beads.

Example 4: Hybridization Properties of the Covalently Conjugated Oligonucleotide.

The ability of the covalently conjugated oligonucleotide to hybridize to complementary DNA or RNA in solution was demonstrated as follows: Pall membrane was derivatized with 2-aminoethanethiol and conjugated with SIAB-Bg5B6n as described above. Discs of the

TABLE 2

Compound % Capping (disulfide formation)

(hydrophobic)	triphenylmethyl mercaptan (TMM)	20%
(cationic)	dimethylaminoethanethiol (DMEA)	66%
(cationic)	2-aminoethanethiol	100%
(hydrophobic)	thiocholesterol	100%
(hydrophylic)	penicillamine	12%

Hybridization assays as described above indicate that the rate of hybridization is increased approximately 1.5-fold when the surface is made hydrophobic with TMM or DMAE.

Example 6A: The Multiple Target Dipstick for Detecting Peridontal Bacteria.

Preparing the multiple target dipstick requires the selection of a semi-rigid plastic stick of polystyrene. The stick is approximately 5 cm long, having two flat sides having a width of between 0.1 and 0.5 cm and a depth of between 0.05 and 0.3 cm.

A commercial preparation of CML Tube coating is diluted with 50% ethanol. The diluted CML Tube Coating is painted upon selected regions of the dipstick. Any excess CML Tube Coating is removed and allow the material is allowed to dry without the use of heat. After drying the carboxy groups of the CML Coating are activated using 1-ethyl-3-(3-diethylaminopropyl)carbodiimide (EDC). The EDC in used in a non-amine containing buffer at 10 to 100 mg/ml at pH 5.0. The reaction is allowed to proceed for 2 minutes at 18° to 25° C and the EDC is then decanted.

Immediately after decanting the EDC add 0.01 to 1 molar cystamine or cysteamine (2-aminoethanethiol) in a non-amine containing buffer at pH 8.0 and allow the reaction to continue for 1 to 24 hours. After appropriate washing steps with phosphate buffered saline followed by water the cystamine is reduced to 2-

sample can be frozen until use. When the assay is ready to begin, 100 ng of an unrelated carrier 24 mer oligonucleotide is added to the sample if the bacterial count is low. 500 ul of sucrose lysis buffer is added comprising: 75% sterile sucrose, 10 mM EDTA, 10 mM EGTA, and 50 mM Tris-HCl at pH 8.0. The sample is vortexed briefly.

Bacterial cells are resuspended in a lysis solution (20 mg/ml lysozyme, 25% sucrose, 50 mM Tris, pH 8, 10 mM EDTA), and incubated at 37°C for 30 minutes. Sodium dodecylsulphate is added to a final concentration of 1-2% and pronase (1 mg/ml) or proteinase K (200 µg/ml) and the solution is incubated 30 min at 37°C. The lysates are extracted twice with phenol:chloroform (1:1, v/v) and then ethanol precipitated. Nucleic acid is pelleted, washed with 70% ethanol, and resuspended at approximately 1 mg/ml in 1x TE (10 mM Tris, pH 8.0, 1 mM EDTA).

The bacterial nucleic acids (.01-10 ng/ml) are then placed into a hybridization solution containing 30% formamide, 0.09 M NaCl, 0.01 M Tris-HCl, 5 mM EDTA, 0.1% SDS, 1X Denhardt's solution at pH 8.0. Oligonucleotides, complementary to the conserved regions of the bacterial rRNA: CACGA(G/A)CTGACGACA(G/A)CCATGC and TACGGNTACCTTGTTACGAC and conjugated to horse radish peroxidase are added to a concentration of .01-.2 µg/ml. Conjugation can be achieved using known techniques. The preferred means for conjugating horseradish peroxidase was provided in U.S. Serial No. 148,258, filed January 25, 1988, and is hereby incorporated by reference herein. The hybridization reactions are permitted to carry on at 42°C for 1 hour and the unhybridized oligonucleotides and nucleic acids are removed by repeated washings with hybridization media minus formamide and SDS. The dipstick is then washed with 0.1 M Na citrate at pH 5.5 and developed in 0.1 M Na citrate, pH 5.5, 0.5 mg/ml 4-methoxy-1-naphthol, 0.02 mg/ml 3-methyl-2-benzo-

containing 0.5% SDS. The filters were then probed with 5 mg/ml of Streptavidin/horseradish peroxidase conjugate in filter wash containing 0.5% SDS. The incubation was for 1 hour at 19°C. The dipsticks were then washed at ambient temperature 3 times with SDS/filter wash and three times with filter was without SDS. The filters were developed as described in Example 7 below.

The results indicated that specific bacterial species (one or all the bacterial species described above) could be detected in a complex mixture of cells and organic material using a multiple site dipstick.

Example 7: Demonstration of the Multiple Target.

A. Dipstick Using Four Specific Oligonucleotides Covalently Immobilized on Pall Membrane

One 24-nucleotide species specific sequence from the hypervariable region of each of the 16s rRNAs from Actinobacillus actinomycetemcomitans, Bacteroides gingivalis, Eikenella corrodens, and Bacteroides intermedius is synthesized possessing a 5'-terminal primary amine hexyl-linker as described in Example 6. Each oligonucleotide is covalently immobilized on a Pall membrane as described in Examples 1-5 above using a Minifold 11 slot blot apparatus (Schleicher & Schuell, Keene, NH). Approximately 0.1 microgram of each type of the 24-mer oligonucleotide is immobilized per slot and each slot is approximately 1 cm in length separated by a distance of 3 cm. The Pall membrane is cut such that each strip of membrane possesses the four different oligonucleotide regions. Each strip is then used in a separate hybridization assay.

The dipsticks are then placed in 1.5 ml solutions containing 30% formamide, 0.09 M NaCl, 0.01 M Tris, pH8.0, 5 mM EDTA, 0.1% SDS, 1X Denhardt's solution and 0.2 ug of a 48-mer synthetic oligonucleotide complementary to the 24 nucleotide sequence immobilized on the membrane as well as a 24 nucleotide length

TABLE 4

Virus	Capture Oligo	Signal Oligo	Synthetic Oligonucleotide	%GC
HPV6	+		3'GGTTGAACCGTTTCGGTCCCCTCC5'	62.5
HPV6		+	3'GCTGCCATAACATACTTCCCAATG5'	45.8
HPV 16/33		+	3'GCTGAGTTTCCACTTCAGTATTGC5'	45.8
HPV 16/33	+		3'CACGTCCTTGAGAAAAAGGATTTC5'	41.6
HPV 18	+		3'GAATGCTCGAAGTCGTCTGCTGAG5'	54
HPV 18		+	3'TCGTCGGGCTGGTAAATGTTGATG5'	50

The signal oligonucleotides are biotinylated using sulfo-NHS-biotin as described in Example 9.

The dipsticks immobilized with different combinations of the three capture oligonucleotides are placed into a hybridization solution containing a mixture of HPV6, 16 and 18 plasmid DNA's (0.01-10 ng/ml), 30% formamide, 0,09M NaCl, 0.01M Tris-HCl, 5mM EDTA, 0.1% SDS, 1X Denhardt's solution at pH 8.0 at 42°C for 1-24 hours and the unhybridized nucleic acids are removed by repeated washing with hybridization media minus formamide and SDS. The dipsticks are now probed with the different biotinylated oligonucleotides in the same hybridization solution replacing a 0.09M NaCl with 0.6M NaCl for 1 to 4 hours. The dipsticks are washed well with 2 X filter wash (0.009 M Tris pH 8.0, 0.18 M NaCl, 2 mM EDTA) and then reacted with streptavidine-horseradish peroxidase conjugate (Zymed Laboratories, Inc., San Francisco, California) 2 µg/ml in 2X filterwash containing 0.1% SDS for 30 minutes. The dipsticks are drained and then washed several times with 0.1% Tween in 1X phosphate buffered saline (PBS, 0.01M phosphate pH 7.4, 0.15M NaCl). The color is developed as described in Example 7.

Intense blue precipitates on selected areas of the dipstick confirm the presence of the different HPV nucleic acids.

TABLE 5

The Kinetic $T_{\frac{1}{2}}$ of Different Solid Supports

Solid Support	$T_{\frac{1}{2}}$
Pall Immunodyne membranes	3-4 hrs
CML on Plastic Sheet	5 min
CML on Polyethylene Frit	10 min
Beads on Polyethylene Frit	10 min
Polyethylene Beads in Solution	2 min

Example 9: The Synthesis of Biotinylated Short Probes.

5'-Aminohexyloligonucleotides at a concentration of 1 mg/ml is reacted with a 100 fold excess of sulfosuccinimidyl-6-(biotinamido) hexonate (NHS-LC-biotin) in 0.1 M carbonate buffer at pH 9.5 for 30 minutes at room temperature. The reaction is monitored by HPLC on a reversed phase C8 300°A Dynamax, Rainin column with a CH_3CN/H_2O mobile phase and if reaction is less than 90% completed additional NHS-LC-biotin is added. The biotinylated oligo is HPLC purified as above as a single peak. The organic phase is removed under reduced pressure and the biotinylated oligos redissolved in distilled water for use in assays.

Example 10: The Use of Polycysteine as an Amplification System for Increasing the Quantity of Capture Oligo on the Surface of the Solid Support.

Poly-S-CBZ-L-cysteine was obtained from Sigma Chemical Company (St. Louis, MO) and dissolved at a concentration of 20 mg/ml in dimethyl formimide and 50 microliter volumes were applied to 0.28 cm squared discs of Pall membrane and incubated at ambient temperature for 15 minutes. The membrane discs were then incubated in 5 ml of concentrated ammonium hydroxide for 30 minutes at 20 degrees C. to de-block the CBZ groups and generate the free sulfhydryl groups. A DTNB assay (see Example 1)

reduced, the filters chemically reduced exhibited a greatly reduced background of bound Streptavidin/HRP. Essentially no color was observed on the chemically reduced capped filters, while the uncapped filters or the capped filters not treated with DTT showed blue color indicating the presence of oxidized 4-methoxynaphthol. Therefore, the cleavage of the disulfide bond leading to the release of 2-aminoethanethiol or thiolcholestrol significantly reduces background to chemical cleansing of the surface of the solid support.

Example 12: The Equalization of Melting Characteristics of Probes with Different GC Content in TEABR.

a) HPV16 Plasmid Immobilized on Nytran Filters

Twenty ng/ml each of two $^{3}2p$ labeled 24 mer probes specific for HPV18 nucleic acid namely HPV18A1B (25% GC content) and HPV18A3B (67% GC content) in hybridization solution containing 0.6 M NaCL 90 mM TRIS pH 8.0, 0.5% SDS, 5x Denharts solution, 30% formamide and 0.1 mg denatured Salmon sperm DNA are hybridized over night at 42°C to HPV16 plasmid DNA immobilized on Nytran filters. The filters are then washed twice with different concentrations of TEABR solution containing 50mM TRIS pH 8.0, 2mM EDTA, 0.1% SDS, ranging from 2.8M to 3.4M in 0.1M increments for 30 minutes at 29°C. The filters are monitored for radioactivity. Similar signals are observed only with the wash in 3.1M TEABR for both probes indicating that the melting temperatures are equalized leading to comparable rates and extents of hybridization.

b) Sandwich Assay Capture of B. gingivalis rRNA

Two 24 mer B. gingivalis probes (Bg5B and Bg8B which differ in G+C content to the extent that a 20° difference in Tm is observed between the two probes) were employed on the "signal" side of the sandwich assay to determine the affects of TEABR in the hybridization solution on the extent of hybridization. In this experiment B. gingivalis rRNA was captured using a

oligonucletide probes complementary to conserved regions of bacterial 16s rRNA (signal probes) were added to a final concentration of 100 nanograms per ml to the lysate and to the 3 M GuSCN lysing solution that was to be used as the diluent. Seven, ten-fold serial dilutions were then made. The solutions were incubated with Nytran® discs which had covalently immobilized 1 microgram of Bg specific oligonucleotide probe (capture probe) for 1 hour at ambient temperature. The solid supports were then washed with SDS/FW (sodium dodecyl sulfate and filtered water) at ambient temperature and then incubated with 10 ng/ml of Strepavidin/Horseradish peroxidase (SA/HRP) conjugate in SDS/FW for 30 minutes at ambient temperature. The solid supports were then washed with SDS/FW, FW (filtered water), and then the presence of peroxidase was determined by incubating the filter with a substrate that formed an insoluble product. The results indicated that a level of sensitivity of 1×10^6 bacterial cells was achieved.

8. A dipstick of claim 1 wherein the nucleic acid probes are covalently bound to the solid surface through spacer arms.

9. A spacer arm of claim 8 derived from a thiol reactive substituent bound to a tethered nucleophilic amine on the 5' ends of the nucleic acid probes, said substituent having the formula:

$$— (CH_2)_n — X — Y$$

where X is -NH- or -NHC:O$(CH_2)_m$NH-, Y is a thiol reactive moiety, m is 2-12 inclusive, and n is 2-12 inclusive.

10. A spacer arm of claim 9 where n is six and X is -NH-.

11. The spacer arm of claim 9 wherein the reactive group upon the thiol-reactive moiety is an α halo-acyl or an α, β-unsaturated carbonyl.

12. The thiol reactive moiety of claim 11 wherein the thiol-reactive moiety is selected from the group comprising haloacetamidobenzoyl and 4-(N-maleimidomethyl)-cyclohexane-1-carbonyl.

13. A dipstick of claim 1 having a solid surface derivatized with sulfhydryl containing moieties.

14. A dipstick of claim 13 wherein the sulfhydryl containing moieties are polymeric compounds having a multiplicity of sulfhydryl groups.

15. A solid surface of claim 14 having proteins conjugated thereto through sulfhydryl bonding.

16. A surface of claim 15 wherein the proteins are selected from the group consisting of bovine serum albumin; casein; and liquid gelatin.

25. A method of claim 24 wherein the nucleic acid probes are bound to a solid surface comprising one of the following group:

a. polystyrene/latex;
b. polystyrene;
c. latex beads;
d. carboxyl modified latex microspheres;
e. carboxyl modified glass; and
f. carboxyl modified teflon.
g. Nytran®.

26. A method of claim 25 wherein the solid surface is comprised of carboxyl modified latex microspheres.

27. A method of claim 26 wherein a solid surface which does not inhibit diffusion of nucleic acid.

28. A method of claim 27 having a solid surface with an average pore size in excess of 100 microns.

29. A method of claim 24 wherein the nucleic acid probes are covalently bound to the solid surface through spacer arms.

30. A method of claim 29 wherein the spacer arms are derived from a thiol-reactive substituent linked to a tethered nucleophilic of amine on the 5' ends of the nucleic acid probes wherein the substituent is of the formula:

$$— (CH_2)_n —X-Y$$

where X is -NH- or $-NHC{:}O(CH_2)_mNH-$, Y is a thiol reactive moiety, m is 2-12 inclusive, and n is 2-12 inclusive.

31. A method of claim 30 where n is six and X is -NH-.

40. A method of claim 24 comprising hybridizing target and probe nucleic acids in a hybridization buffer comprising an ammonium salt selected from the group consisting of trialkylammonium salt and tetraalkylammonium salt wherein the alkyl groups are the same or different and are comprised of between 1 and 3 carbon atoms inclusive.

41. A method of claim 40 wherein the trialkylammonium salt is triethylammonium chloride.

42. A method of claim 40 wherein the salt is a tetraalkylammonium salt.

43. A method of claim 42 wherein the tetra-alkylammonium salt is tetramethylammonium chloride or tetraethylammonium chloride.

44. A method of claim 40 wherein the salts are selected from the group comprising acetate, iodide, perchlorate, thiocyanate, chloride and bromide.

45. A method of claim 42 wherein the concentration of alkylammonium salt is between about 2 M and 3.5 Moles.

46. A method for conducting a nucleic acid hybridization assay using nucleic acid probes covalently bound to a solid surface, the method comprising (a) modifying the surface of solid support in a nucleic acid hybridization assay by covalently binding a surface-modifying moiety through a covalent bond selected from the group consisting of disulfide linkage and thiol ether; (b) contacting the solid support with a hybridization medium containing target nucleic acid; (c) removing target nucleic acid not hybridized to the

the surface through disulfide bonds; (b) releasing the proteins through thiol reduction after step (a); and (c) detecting hybridization between target and probe nucleic acids.

53. A method of claim 52 wherein the proteins linked to the surface are selected from the group comprising bovine serum albumin; casein; or liquid gelatin.

54. A method of claim 52 wherein the reactive surface comprises a solid surface comprising a multiplicity of discrete regions having nucleic acid probes covalently bound thereto.

55. A method of claim 54 wherein the nucleic acid probes are bound to a solid surface comprising one of the following group:

a. polystyrene/latex;
b. polystyrene;
c. immobilized latex beads;
d. carboxyl modified latex microspheres;
e. carboxyl modified glass; and
f. carboxyl modified teflon.
g. Nytran®.

56. A method of claim 55 wherein the surface is comprised of carboxyl modified latex microspheres.

57. A method of claim 52 wherein the nucleic acid probes are covalently bound through spacer arms.

58. A method of claim 57 wherein the spacer arms are derived from a thiol reactive substituent linked to a tethered nucleophilic amine on the 5' ends of the nucleic acid probes wherein the substituent is of the formula:

67. A method of claim 66 wherein the nucleic acid probes are complementary to sequences of human papilloma virus DNA.

68. A method for conducting multiple target nucleic acid hybridizations in a sandwich assay format comprising hybridizing a multiplicity of nucleic acid probes to different target nucleic acids using a hybridization buffer comprising an amount of a salt having an anion and a cation wherein the cation is selected from the group comprising trialkylammonium and tetraalkylammonium wherein the alkyl groups are the same or different and are comprised of between 1 and 3 carbon atoms inclusive, said amount of salt effective to reduce the effect of variations in G:C and A:T content between the probes and the target nucleic acids when hybridizing into duplexes.

69. A method of claim 68 wherein the trialkylammonium salt is triethylammonium chloride.

70. A method of claim 68 wherein the salt is a tetraalkylammonium salt.

71. A method of claim 70 wherein the tetra-alkylammonium salt is tetramethylammonium chloride or tetraethylammonium chloride.

72. A method of claim 68 wherein the anions are selected from the group comprising acetate, iodide, perchlorate, thiocyanate, chloride and bromide.

73. A method of claim 70 wherein the concentration of alkylammonium salt is between about 2 M and 3.5 Moles.

probes to target probes and then detecting the hybridization of labeled signal probes.

80. A method of claim 79 wherein the assay is a sandwich assay.

81. A method of claim 79 wherein the detecting step involves comparing the signal strength of the assay to standardized signal levels.

82. A method of claim 80 wherein the target polynucleotide is derived from bacteria.

83. A method of claim 81 wherein the target polynucleotide is derived from ribosomal RNA.

84. A kit for conducting nucleic acid sandwich assays comprising containers containing signal probes in predetermined ratios of labeled to unlabeled probes.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03378

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C12Q 1/68

U.S. Cl.: 435/6

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
U.S.	435/6,174,291,805,810 436/501,172,810 536/27 935/2,78,87

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	US,A, 4,391,904 (LITMAN ET AL.) 05 JULY 1983 (05.07.83), see entire disclosure.	1-84
Y	Nucleic Acids Research, Volume 15, Number 13, published in 1987 (IRL Press Limited, Oxford, England), S. Ghosh et al., "Covalent attachment of oligonucleotides to solid supports," pages 5353-5372, see especially the INTRODUCTION on pages 5353-5355.	1-8,17-29, 36-39,47, 55-57,63-67
X,P	US,A, 4,775,619 (URDEA) 04 OCTOBER 1988 (04.10.88), see Figure 1, DESCRIPTION OF THE SPECIFIC EMBODIMENTS (columns 2-11), and especially column 11, lines 20-38.	1
Y,P		2-14,17-34 36-39,46-51, 54-67,74-84

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
13 DECEMBER 1989 (13.12.89)	04 JAN 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	ARDIN MARSCHEL

Form PCT/ISA/210 (second sheet) (Rev. 11-87)